Uncouplers and the molecular mechanism of uncoupling in mitochondria

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ABSTRACT Uncouplers are molecules with protonophoric and ionophoric capabilities that mediate coupled cyclical transport of cations—a transport that takes precedence over all other coupled processes. Uncouplers form cation-containing complexes with electrogenic ionophores that potentiate cyclical transport of cations. The molecular mechanism of uncoupling sheds strong light on the mechanism of coupling.

Electron flow in mitochondria can drive synthesis of ATP, accumulation of cations (K⁺, Mg²⁺, Ca²⁺), or transfer of hydrogen from NADPH to NAD⁺. Uncouplers sever the link between the driving reaction and the driven reactions without suppressing the rate of electron flow (1–3). The same effect of uncoupler on coupled reactions is observed when the driving reaction is hydrolysis of ATP rather than electron flow. This phenomenon of uncoupling is the most characteristic feature of mitochondrial energy coupling, and, although discovered some 30 years ago (3), its molecular mechanism has been an enigma. One valuable hint about the action of uncouplers has come to light. Under the conditions in which uncoupling takes place, all protonic and cationic gradients are abolished (4, 5)—an indication that uncouplers are carriers of both protons and cations.

There are 20 to 30 different molecular species that have in common the property of suppressing all coupled processes whether driven by electron flow or hydrolysis of ATP and the property of being equally effective in these two respects regardless of the substrate used for electron flow or the nature of the coupling site (6, 7). They differ only in respect to the concentration at which uncoupling is maximal. The most efficient uncouplers such as SF6847 can uncouple in the nanomolar range, whereas the less efficient uncouplers such as penta-chlorophenol and 2,4-dinitrophenol are effective only in the millimolar range.

Uncoupling by the combination of valinomycin and nigericin in presence of K⁺

It has been known for some time that the combination of two ionophores (valinomycin and nigericin) can uncouple oxidative phosphorylation in the same fashion as do classical uncouplers but only in the presence of K⁺ (8, 9). We considered this correspondence of action as an invaluable clue since it has already been established that the combination of valinomycin and nigericin induces cyclical transport of K⁺ coupled to electron flow (Fig. 1). The critical question was whether this combination of ionophores could duplicate all the known properties of uncouplers. The data of Table 1 clearly show that the correspondence is complete in all respects.

On the basis of this correspondence we formulated the hypothesis that uncouplers are inducers of coupled cyclical transport of cations and that this particular coupled process takes precedence over all other coupled processes such as coupled ATP synthesis, active transport of cations, and energized transhydrogenation. It would be predictable that, in order to mediate cyclical transport of cations: (i) uncouplers must be both ionophores and protonophores; (ii) uncoupling should depend upon the availability of cations for cyclical transport; and (iii) the concentration of uncoupler required for maximal uncoupling should be at least equal to the concentration of the complexes of the electron transfer chain (this equality has already been demonstrated for valinomycin (16) in the release of respiration of Complex IV by the combination of valinomycin, nigericin, and K⁺). One of the key predictions of the cyclical cation transport hypothesis—the elimination of all gradients—has already been demonstrated (see Table 1).

Uncouplers as protonophores and ionophores

The prevailing dogma that uncouplers uncouple by virtue of collapsing a proton gradient has spawned a vast literature attesting to the protonophoric capability of uncouplers (17–19), and hence this capability has been most thoroughly documented and verified. All uncouplers, without exception, can dissociate a proton in their uncharged state and can take up a proton in their charged state in the pH range of uncoupler action. In general, the best uncouplers have a pK above pH 6 and the less efficient uncouplers have a pK below pH 6.

Elsewhere we have presented evidence for the ionophoric capability of all uncouplers for both monovalent and divalent cations (6). Although there can be no question from the available data that uncouplers are bona fide ionophores, the conditions under which this capability is demonstrable rule out the possibility that uncouplers can function as ionophores in the physiological range of pH. It is only when the pH of the aqueous phase in a two-phase system is in the range of 8–10 that the ionophoric capability of uncouplers is significant. The important point to be emphasized, however, is that the capability is present.

requirement of cations for the action of uncouplers

The general impression in the literature has been that uncoupling is not a cation requiring process, although there have been occasional reports to the contrary (20, 21). What was not appreciated by other investigators of the uncoupling phenomenon and what we had to learn the hard way was that well-coupled mitochondria contain bound Ca²⁺ and Mg²⁺ in more than sufficient amounts to mediate the action of uncouplers. Table 2 contains data on the average values for bound Ca²⁺ and Mg²⁺ in well-coupled beef heart mitochondria and the degree to which the complement of bound divalent ions can be depleted by various reagents and experimental conditions. We have found that the acidic ionophore A-23187 is the reagent par excellence for depletion of coupled mitochondria to the point at which a cation requirement for uncoupling is demonstrable (22). The action of A-23187 can be duplicated by EDTA (23)
Table 1. Parallelism of the action of uncoupler and the combination of valinomycin and nigericin in presence of K⁺ on coupled mitochondrial processes

<table>
<thead>
<tr>
<th>Mitochondrial process</th>
<th>Units of change</th>
<th>Control</th>
<th>Plus uncoupler</th>
<th>Plus valinomycin and nigericin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Release of respiratory control</td>
<td>ng atoms O</td>
<td>79</td>
<td>424</td>
<td>394</td>
</tr>
<tr>
<td>Release of latent ATPase activity</td>
<td>nmol ATP hydrolyzed</td>
<td>62</td>
<td>830</td>
<td>800</td>
</tr>
<tr>
<td>Coupled ATP synthesis</td>
<td>nmol Pi esterified</td>
<td>612</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Active transport of Ca²⁺</td>
<td>ng atoms Ca⁺⁺</td>
<td>732</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Energized transhydrogenation</td>
<td>nmol NADH formed</td>
<td>76</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Reversed electron flow</td>
<td>nmol NADH formed</td>
<td>135</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Energized release of protons</td>
<td>ng atoms H⁺ released at equilibrium</td>
<td>33</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The various measurements were carried out at 30° with beef heart mitochondria according to the following methods: release of respiratory control, Hunter et al. (10); release of latent ATPase activity, Sadler et al. (11); coupled ATP synthesis, Southard and Green (12); active transport of Ca²⁺, Southard and Green (12); energized release of protons, Southard et al. (13); energized transhydrogenation, MacLennan et al. (14); reversed electron flow, Ernster and Lee (15).

* All are per min/mg of mitochondrial protein, except energized release of protons.

Fig. 1. Cyclical transport of K⁺ by the combination of valinomycin and nigericin. O, valinomycin; Nig⁻, charged form of nigericin; Nig⁺, protonated form of nigericin; H⁺, hydrogen originating from the substrate for electron transfer complex; A, final acceptor of the electron transfer chain. Charge separation in the electron transfer complex (separation of H⁺ and e⁻) is paired to the valinomycin-mediated separation of Nig⁻ into Nig⁺ and Nig⁺⁺. Charge elimination in the electron transfer complex is paired with A to K⁺ with Nig⁻.

The mitochondrial suspensions were finally washed in 0.25 M sucrose containing 1 mM Tris-HCl at pH 7.4 before analyses by atomic absorption by the method of Southard and Green (12). For details of the separation of mitochondria in the various states, see Hunter et al. (10) for the preparation of orthodox mitochondria, legend of Table 3 for the preparation of A-23187-treated mitochondria, and legend of Table 4 for the mitochondria exposed to EDTA.
Table 3. Cation-dependence of uncoupler action in beef heart mitochondria depleted of divalent metals with A-23187

<table>
<thead>
<tr>
<th>Addition to mitochondria</th>
<th>Rate of oxidation of durohydroquinone, ng atoms O/min per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without uncoupler</td>
</tr>
<tr>
<td>None</td>
<td>223</td>
</tr>
<tr>
<td>KCl (10 mM)</td>
<td>264</td>
</tr>
<tr>
<td>KCl (100 mM)</td>
<td>619</td>
</tr>
<tr>
<td>NaCl (100 mM)</td>
<td>619</td>
</tr>
<tr>
<td>Tris·HCl (100 mM)</td>
<td>219</td>
</tr>
</tbody>
</table>

Heavy beef heart mitochondria were exposed to A-23187 (0.5 nmol/mg of protein) for 30 min at 30°C in 0.25 M sucrose/10 mM Tris·HCl, pH 7.6/1 mM EDTA. The mitochondria were then washed twice in 0.25 M sucrose/10 mM Tris·HCl, pH 7.6, and resuspended in 0.25 M sucrose/1 mM Tris·HCl, pH 7.5. In the measurement of respiration by the oxygen electrode method, the substrate was durohydroquinone (1.3 mM) and the uncoupler was mClCCP (2 μM). The concentration of mitochondrial protein was 0.5 mg/ml. The experiment was carried out at 30°C.

Valinomycin can potentiate active transport of K⁺ in mitochondria, and this active transport is suppressed by addition of uncoupler (24, 25). We could then use valinomycin as a model for the interaction of uncouplers with intrinsic electronegic ionophores. The combination of valinomycin and uncoupler (SF-6847) increased the partition of Rb⁺ from an aqueous to an organic phase (toluene/butanol, 70:30) by a factor of 27 or more as compared to the partition induced by valinomycin or uncoupler alone. A Pressman cell experiment (26) confirmed that the combination of valinomycin and uncoupler mediated transport of Rb⁺ through an organic phase under conditions in which neither valinomycin nor SF-6847 alone could mediate a significant degree of transport (Fig. 2). We have tested synthetic neutral ionophores that can induce active transport of monovalent cations (corn ether and nactin) and found that these show synergistic action with uncoupilers comparable to that of valinomycin (5 to 10-fold increase in the degree of partition or the rate of transport of Rb⁺). Furthermore, we have found that SF-6847 can be replaced by other uncoupilers in these synergistic reactions.

It is a reasonable inference that underlying the synergistic action is a 1:1:1 complex of electronegic ionophore (EI), monovalent cation (Me⁺), and uncoupler (U⁻), represented by EI-Me⁺-U⁻. The cation is coordinated with polar groups in both the electronegic ionophore and the uncoupler. A simple experiment provided strong support for this postulated composition of the complex. The combination of valinomycin and uncoupler, each at a concentration of 100 nmol/ml of the aqueous phase, induced the partition of 87 nmol of Rb⁺ in the organic phase. When the concentration of valinomycin, but not of uncoupler, was increased or when the concentration of uncoupler, but not of valinomycin, was increased, the partition of Rb⁺ remained at the value corresponding to equimolar concentrations of the two reactants.

Does the same synergism apply to electronegic ionophores for divalent cations? To test this possibility it was first necessary to find ionophores that could induce active transport of divalent cations in mitochondria. We have found that beauvericin and Triton X-100 could function in that capacity in mitochondria exposed to a level of ruthenium red that inactivates the intrinsic electronegic ionophore for Ca²⁺. Under these conditions, beauvericin and Triton X-100, each at about 0.02 mM concentration, induced active transport of Ca²⁺ in presence of appropriate anions such as inorganic phosphate. The synergistic effect of beauvericin and uncoupler in mediating transport of Ca²⁺ was demonstrable in a Pressman cell experiment and was

Table 4. Restoration of uncoupler-mediated release of respiration by addition of Mg²⁺ or Ca²⁺ to heavy beef heart mitochondria depleted of divalent metal cations by exposure to EDTA

<table>
<thead>
<tr>
<th>Type of mitochondria</th>
<th>Additions</th>
<th>Rate of respiration, ng atoms O/min per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without uncoupler</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
<td>377</td>
</tr>
<tr>
<td>Exposed to EDTA</td>
<td>0</td>
<td>311</td>
</tr>
<tr>
<td>Exposed to EDTA</td>
<td>CaCl₂ (1 mM)</td>
<td>754</td>
</tr>
<tr>
<td>Exposed to EDTA</td>
<td>MgCl₂ (1 mM)</td>
<td>622</td>
</tr>
</tbody>
</table>

Heavy beef heart mitochondria suspended in 0.25 M sucrose/1 mM Tris·HCl at a final protein concentration of 10 mg/ml were supplemented with the sodium salt of EDTA (3.5 mM) and the pH was adjusted to 7.9. The suspension was incubated for 10 min at 25°C and then cooled on ice before centrifugation. The pellet was resuspended in the original medium (no EDTA added). Durohydroquinone (1.3 mM) was used as substrate; mClCCP (2 μM) was the uncoupler.
indistinguishable from that shown in Fig. 2 for the valinomycin-uncoupler synergism. Thus, we may conclude that the synergistic effect of uncoupler and electrogenic ionophore is general whether the electrogenic ionophore acts on monovalent or divalent cations [see (27) for the electrogenic nature of beavercin].

Molecular mechanism of uncoupler-induced cyclical transport of cations

Fig. 3 shows formulations in terms of the paired moving charge model of the mechanism of uncoupler-induced cyclical transport of monovalent and divalent cations. Strictly speaking, both the cation and the uncoupler are cyclical transported according to the formulations. In cyclical transport of monovalent cations, the cycling of the uncoupler is nonelectrogenic but in cyclical transport of divalent cations, the cycling of the uncoupler has both an electrogenic and a nonelectrogenic step. It has been established by Moyle and Mitchell (28) and others (29) that the electron drives the transport of one calcium ion across the membrane. This 1:1 stoichiometry would require the cotransport of a singly charged negative species with Ca$^{2+}$ in order to satisfy the requirements of charge neutrality.

The molar relationship between the concentration of uncoupler and the concentration of the electron transfer complex

According to the formulations shown in Fig. 3, there should be a 1:1 molar relationship between the electron and the uncoupler in cyclical transport of monovalent cations and a 1:2 molar relationship in cyclical transport of divalent cations. In untreated mitochondria suspended in 0.25 M sucrose buffered with 1–10 mM Tris-HCl, the uncoupler-mediated release of respiration was entirely dependent on bound divalent metals. The stoichiometry for the electron and the uncoupler was approached at the point of maximal release of respiration (Fig. 4). Because both monovalent and divalent cations are available to the uncoupler, the theoretical ratio should be 1:1 and 1:2. The uncouplers tested that showed close to theoretical stoichiometry (SF-6847 and mCICCP) were among the most efficient uncouplers. Such titrations are difficult to carry out with the weaker uncouplers because in general their partition is greatly in favor of the aqueous phase. The antimycin titer is taken as a measure of the concentration of each of the electron transfer complexes (30). This comes to a value of 0.21 nmol/mg of protein. Because the oxidation of durohydroquinone by molecular oxygen involves two complexes (III and IV), the concentration of the electron transfer complexes reacting with uncoupler is assumed to be 0.42 nmol/mg of protein. There have been reports in the literature that less uncoupler is required than is stoichiometric with the electron (31). In such determinations, the substrates selected for driving electron transfer did not fully exploit the capacity of the electron transfer chain for electron flow.

Uncoupler-mediated release of ATPase activity

All the effects that have been documented for uncoupler-mediated release of electron flow apply as well to the effects of uncouplers on ATP-driven coupled reactions. These effects include the cation requirement for uncoupling and the 1:1 or 1:2 molar relationship between ATPase and uncoupler at the point of maximal release of ATP activity.

Synergism of intrinsic electrogenic ionophore and uncoupler

Blondin et al. (32, 33) have isolated from beef heart mitochondria a neutral peptidic ionophore that can induce active transport of monovalent cation in untreated mitochondria. The electrogenic influx rate induced by the isolated peptidic ionophore is a small fraction (<10%) of the nonelectrogenic efflux rate observed when active transport is terminated by addition
of uncoupler. This 10-fold or more augmentation in the efflux rate of $K^+$ induced by the combination of uncoupler and electrogenic ionophore is another manifestation of the synergistic action of these two species. That this is a synergistic action is confirmed by the efflux rate when active transport is inhibited by addition of antimycin. In this instance, the efflux rate is equal to the influx rate.

Direct demonstration of uncoupler-induced cyclical cation transport

When beef heart mitochondria under anaerobic conditions in a medium containing catalase, $K^+$ (2 mM), and durohydroquinone as electron transfer substrate were pulsed with oxygen, a spike of proton release followed by proton uptake and an inverse spike of $K^+$ uptake followed by $K^+$ release were observed with a pH- and a $K^+$-sensitive electrode, respectively, measured simultaneously. The proton and $K^+$ spikes lasted as long as the available oxygen introduced in the pulse (20–30 sec). When uncoupler was added to such a test system in increasing amounts, the spikes became sharper and of shorter duration (1–3 sec at 30°C). The duration of such proton and $K^+$ spikes induced by oxygen pulsing in presence of uncoupler was far less than the time required for complete utilization of oxygen (a 20- to 50-fold discrepancy). What this indicates is that, even in presence of excess uncoupler, there is a temporary imbalance between the influx and efflux rates for protons and $K^+$. This imbalance visualized by the spikes constitutes direct evidence for cyclical cation transport mediated by uncoupler.

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